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Response: Event-related brain dynamics – unifying brain electrophysiology

Scott Makeig

In large part, the study of brain electrophysiology now consists of nearly distinct fields: averaged event-related potentials (ERPs), the ongoing electroencephalogram (EEG), local field potentials (LFPs) and intracellular dynamics (most commonly, spike rate or timing) (Fig. 1). Of these, the two extreme subfields (ERPs and single-cell spike histograms) have been dominant, isolated from each other by differences in spatial scale, recording subjects and, in part, by modeling based on simple averaging methods. Our recent paper in *Science* [1] points out the conceptual impoverishment that results in the case of scalp ERPs, and demonstrates how more adequate analysis of single trials can find dynamic consistencies between features of EEG-trial averages (i.e. ERPs) and event-related changes in the ongoing EEG signals. Meanwhile, other laboratories are exploring links between the timing of neuronal spikes and the dynamics of local-field potentials [2]. Connections between local field potentials and scalp EEG signals are still poorly understood and deserve further study, in which independent component analysis (ICA) [1,3] might also prove useful.

Penny *et al.* [4] rightly place our recent paper on event-related EEG dynamics into the broader context of event-related brain dynamics, as studied in nonlinear dynamic-systems theory and at the level of groups of interconnected neurons. For neuroscience, the study of field dynamics has three

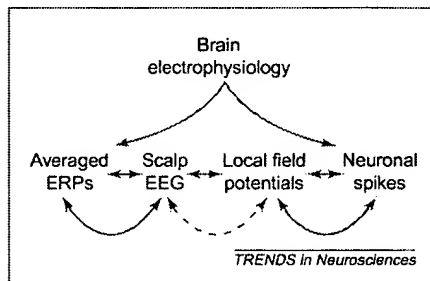


Fig. 1. Neuroscience has largely ignored relationships between four linking subfields of brain electrophysiology. Averaged event-related potentials (ERPs) (recorded from the human scalp) and single-neuron spike histograms (recorded from animals) have dominated, each largely isolated from the other by ignorance of their relationships to ongoing potentials recorded within the brain or on the scalp. This bifurcation has been nurtured by focus on the results of simple response-averaging methods (scalp ERPs, spike histograms). More adequate modeling methods are now demonstrating intimate relationships between all four electrophysiological domains during active cognitive processing.

advantages over single-cell spike train analysis. First, each recorded field signal indexes important (although still poorly understood) physiological processes within neuropile that are largely invisible in, but influence, spike recordings. Second, it is now possible to record fields simultaneously at as many as hundreds of sites over most of the brain, to separate the resulting data into independent and/or transiently dependent signals, and to study the relationship of dynamic changes in these signals and their interrelations to behavior and experience. Third, recent results complement longstanding

observations that distinct changes in the dynamics of brain fields accompany changes in attention [5], memory [6] and awareness [7], strongly suggesting that field dynamics play a role in top-down cognitive processing [8], of which the physiology is still largely mysterious.

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Scott Makeig

Swartz Center for Computational Neuroscience, Institute for Neural Computation, University of California San Diego, La Jolla, CA 92093-0961, USA.
e-mail: smakeig@ucsd.edu

Techniques & Applications

Cyclic amplification of protein misfolding: application to prion-related disorders and beyond

Claudio Soto, Gabriela P. Saborio and Laurence Anderes

Diverse human disorders, including the majority of neurodegenerative diseases, are thought to arise from the misfolding and aggregation of protein. We have recently described a novel technology to amplify cyclically misfolded proteins *in vitro*. This

procedure, named protein misfolding cyclic amplification (PMCA), is conceptually analogous to DNA amplification by PCR and has tremendous implications for research and diagnosis. The PMCA concept has been proved on the amplification of prions

implicated in the pathogenesis of transmissible spongiform encephalopathies. In this article we describe the rationale behind PMCA and some of the many potential applications of this novel technology.

The biological activity of a protein depends of its correct folding in the native conformation. During protein folding, polypeptide chains form a defined three-dimensional structure, as directed by their amino acid sequences. This process is precisely regulated and quality controlled by cellular machinery, such as chaperone proteins and proteases [1,2]. Over the past few years it has become increasingly clear that a hallmark of several diverse diseases is the misfolding of an otherwise normal protein. These diseases are now grouped together under the name of protein conformational disorders (PCD) [3–8]. This group includes most of the neurodegenerative diseases [e.g. Alzheimer's disease, transmissible spongiform encephalopathies (TSE), Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease], and also several systemic disorders (e.g. diabetes type II, serpin-deficiency disorders, haemolytic anaemia, cystic fibrosis, dialysis-related amyloidosis and >15 other, less well-known, diseases) [3,5,8].

'PMCA offers the possibility of amplifying the amount of PrP^{Sc} in a sample, making its detection by existing methods easier.'

TSE are perhaps the best studied of the PCD. They are also known as prion-related disorders, and are fatal neurodegenerative diseases affecting humans and other mammals. Although TSE are rare, their unique mechanism of transmission, and the concerns generated by the recent appearance of variant Creutzfeldt–Jakob disease (vCJD) [which has been linked to consumption of meat contaminated with bovine spongiform encephalopathy (BSE)], has put prions in the spotlight [9]. TSE are the only PCD that have an infectious origin. Although the nature of the infectious agent in TSE has been the center of passionate controversy [10], there is strong evidence that protein misfolding has a crucial role in TSE pathogenesis [9,11]. The most widely accepted hypothesis proposes that the misfolded prion protein (PrP^{Sc}) is not only the most likely cause of the disease, but also the sole component of the infectious agent [11]. PrP^{Sc} replicates in the body by inducing transformation of the natively folded prion protein (PrP^C) into the pathological version [12]. During this process, each PrP^{Sc} molecule can transform up to several

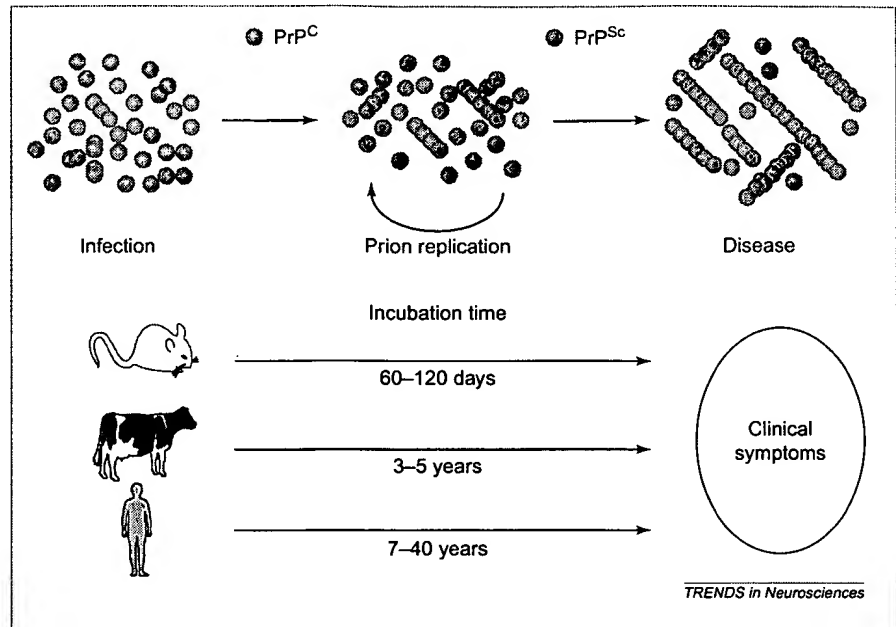


Fig. 1. The prion replication process during the pathogenesis of TSE. At the time of infection, minute quantities of PrP^{Sc} contact large concentrations of PrP^C in the host. During the long and silent incubation period (as illustrated, the length of which depends on host species), PrP^{Sc} molecules replicate, at the expense of PrP^C, to reach a high concentration in the brain – the moment at which clinical symptoms appear. The crucial role of prion replication in the disease is supported by the findings of Weissmann and co-workers that animals devoid of PrP^C are immune to infection [37].

millions of PrP^C molecules. However, this takes a relatively long time – from many months to several years, depending on the species (Fig. 1).

Cyclic amplification of protein misfolding: the rationale behind a novel concept

The replication of the conformation of a protein, leading to changes in the activity of the protein and the phenotype of the individual, is a revolutionary concept in biology: until recently, it was believed that only nucleic acids were capable of replication and transmission of biological information. The self-replication capability of DNA has been used to amplify *in vitro* pieces of DNA by PCR. This amplification process allows synthesis of large amounts of a specific DNA starting with minute (frequently undetectable) amounts of template. A question that has been in the mind of many biochemists is how to develop a system conceptually analogous to PCR that can amplify the properties and activity of proteins. The recognition of proteins with the capability to propagate their biological properties (e.g. prion proteins) opens a new possibility to develop such a system.

We have recently reported a new technology named protein misfolding cyclic amplification (PMCA), which can

multiply minute quantities of PrP^{Sc} at the expense of large amounts of PrP^C [13]. Caughey and co-workers reported previously the cell-free conversion of purified PrP^C in the presence of excess of purified PrP^{Sc} [14]. The reaction has been described in a variety of conditions and has been useful in both understanding the mechanism of prion replication and identifying compounds that interfere with this process [15–18]. However, the yield of this conversion is low, as an excess of PrP^{Sc} is required to convert minimal amounts of PrP^C. To increase the efficiency of prion replication *in vitro*, and to preserve the capability of PrP^{Sc} to convert large quantities of PrP^C, we analyzed the mechanism of prion replication with the aim of mimicking this process *in vitro*.

Although the detailed mechanism of prion replication is not understood fully, it seems to involve a close interaction between the two protein isoforms, facilitated by an as yet unidentified chaperone-like protein [12]. PrP^{Sc} has been described as an oligomer of variable size that can form large fibrillar aggregates resembling the amyloid fibrils observed in Alzheimer's disease and other amyloid-related disorders [19,20]. The comparison of prion conversion with the process of amyloidosis provides a model for the transition of PrP^C to PrP^{Sc},

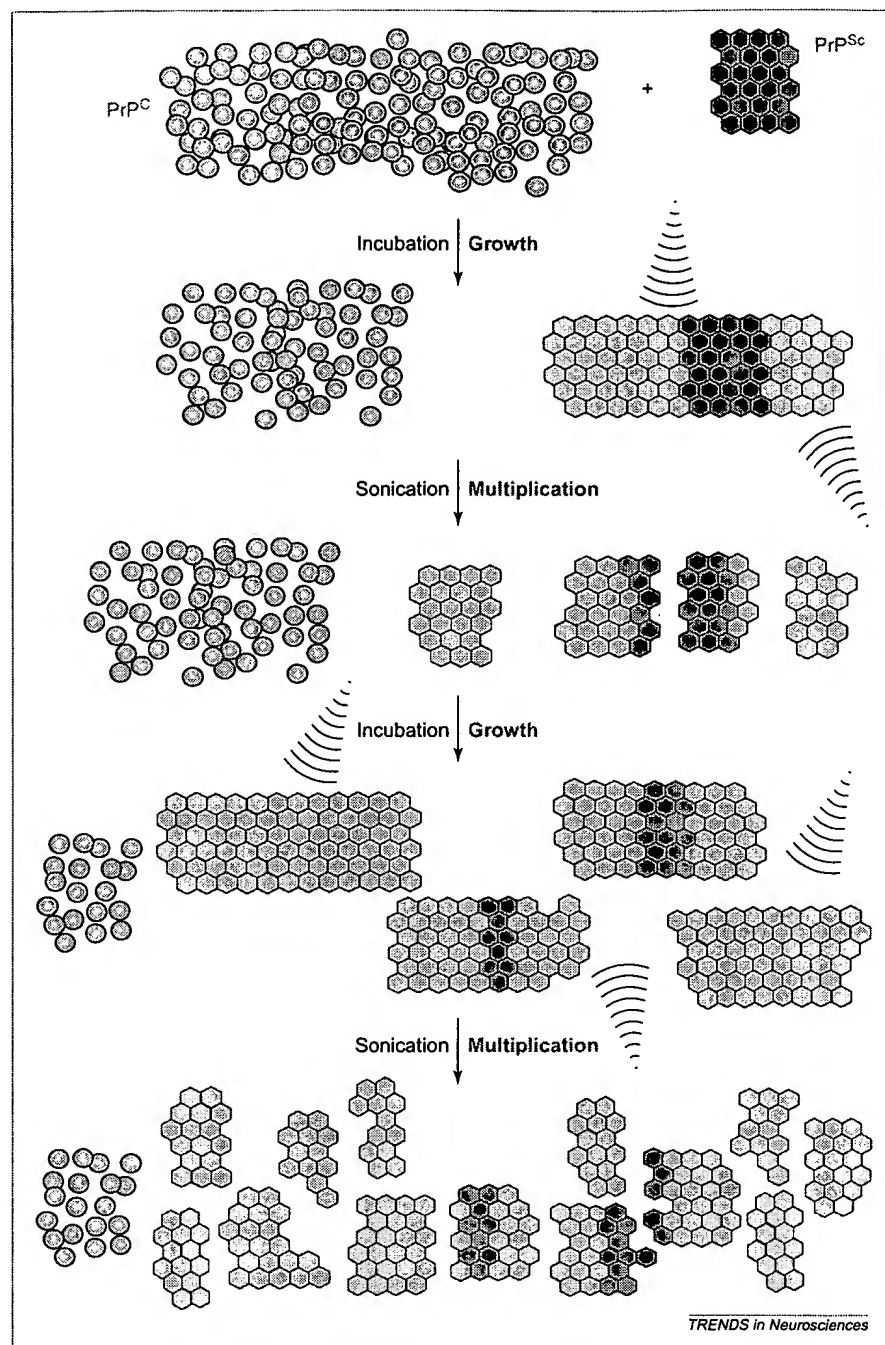


Fig. 2. Protein misfolding cyclic amplification (PMCA). Cyclic amplification consists of subjecting a sample containing minute quantities of PrP^{Sc} and a large excess of PrP^C to cycles of incubation and sonication. During incubation, PrP^{Sc} aggregates grow by converting and incorporating PrP^C into the polymer. Sonication breaks down large aggregates into many smaller pieces to multiply the number of converting units (nuclei). PMCA cycles can be repeated as many times as needed to amplify undetectable quantities of PrP^{Sc} to levels that are easily detected.

of two phases (Fig. 2). During the first phase, a sample containing minute amounts of PrP^{Sc} and a large excess of PrP^C is incubated to induce growth of PrP^{Sc} polymers. In the second phase the sample is subjected to ultrasound to break down the polymers, multiplying the number of converting units (nuclei). In this way, after each cycle the number of seeds is increased in an exponential fashion. The cyclic nature of the system permits the use of as many cycles as required for detection of PrP^{Sc} in a particular sample.

Using the hamster prion strain 263K, we reported proof-of-concept experiments that showed dramatic amplification of minute quantities of PrP^{Sc}, in the presence of a large excess of PrP^C, from healthy hamster brain homogenate [13]. In addition, we demonstrated a clear increase in sensitivity for PrP^{Sc} detection by western blot, and an exponential relationship between the intensity of the PrP^{Sc} signal and the number of amplification cycles [13]. It remains to be documented whether PMCA can be used to amplify PrP^{Sc} from other species or whether it will be useful for diagnosis.

PMCA requires a PrP^C substrate, which must be in high molar excess compared to the amount of PrP^{Sc} for conversion. The method also requires additional cellular factors. Because brain is the target organ for TSE and also the tissue where most of the conversion takes place, we used healthy brain homogenate as a source of PrP^C substrate and conversion factors. The conditions of the solution were selected to be close to the physiological environment in terms of pH and ionic strength, to avoid affecting the interactions between the different components. A low concentration of detergents [0.5% Triton X-100, 0.05% SDS (sodium dodecyl sulphate)] was also included, to avoid protein insolubility and to minimize nonspecific interactions.

PrP^C in the sample does not acquire protease resistance (i.e. is not converted

in which the pathological protein could act as a 'seed' to recruit molecules of partially misfolded PrP^C, stabilizing their misfolding by incorporating them into the oligomer [19,21] (Fig. 1). Thus, the PrP^{Sc} oligomer is elongated at the ends as new molecules of PrP^C are converted and incorporated. This model is supported by mathematical modeling studies [22,23], quantitative data obtained from *in vitro* conversion experiments [19] and by the morphological characterization of prion

aggregates as unbranched polymers with a relatively constant diameter [24,25]. The kinetics of such nucleated conformational conversion is limited by the number of seeds present in the sample [21,22]. This rate-limiting process might explain, in part, the long period of time needed *in vivo* to generate a concentration of PrP^{Sc} high enough to trigger neurodegeneration.

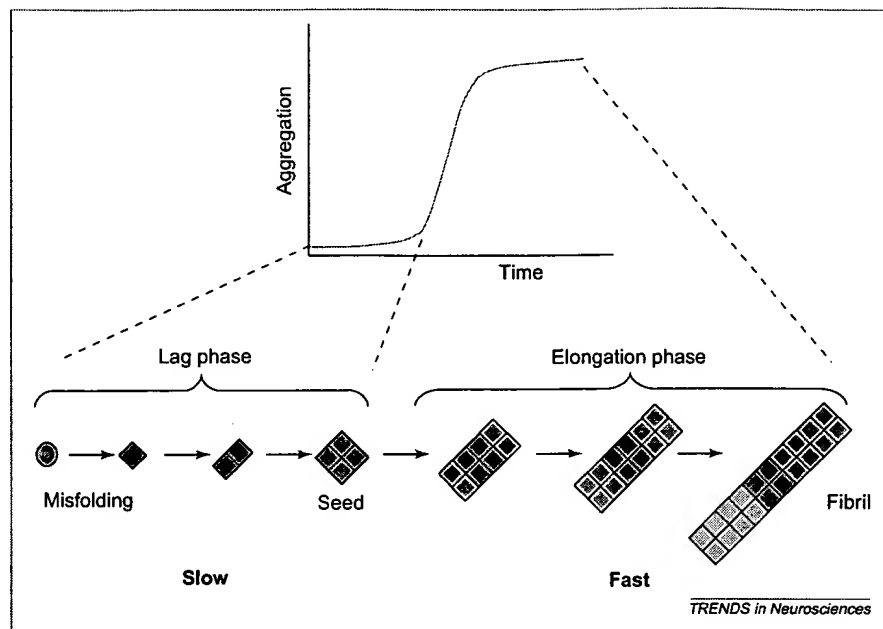
PMCA consists of cycles of accelerated prion replication. Each cycle is composed

Fig. 3. Seeding–nucleation process of amyloid formation. It has been shown that amyloid fibril formation follows a crystallization-like process and that the limiting step is the formation of seeds to further direct aggregation [21,35]. During the lag phase, oligomeric nuclei are formed in a slow process that involves misfolding of the protein and unfavorable intermolecular interactions. Once seeds are formed, a much more rapid phase of elongation results in fibril formation.

to PrP^{Sc}) by PMCA if a template of PrP^{Sc} is not present [13]. In the same way, the PrP^{Sc} initial signal is not modified with the cycles of incubation and sonication in the absence of an adequate substrate (PrP^C) [13]. Although the method seems very simple, several technical considerations have to be observed to reach high levels of amplification. For example, storage and repeated freezing–thawing of normal brain dramatically decrease the efficiency. This is also the case when normal brain homogenate is centrifuged at a speed >1000 rpm for 1 min. In addition, the parameters of sonication and the time between sonication steps have to be carefully optimized for PrP^{Sc} from different species or strains, to adapt to their distinct biochemical properties.

Applications of PMCA in prion research

The cyclic amplification of prions *in vitro* opens many opportunities for understanding better the biology of the infectious agent, for identification and evaluation of novel therapeutic targets and for practical applications in the diagnosis of TSE. The development of tests that can effectively identify animals and people incubating different forms of TSE is a top priority [26,27]. PrP^{Sc} is the only validated surrogate marker for the disease [11,27]. The definitive diagnosis of TSE is based on the demonstration of PrP^{Sc} in brain tissue of the affected host. The problem is that PrP^{Sc} is abundant in the brain only at late stages of the disease. However, several lines of evidence indicate that prions are also present in minute amounts in peripheral tissues, such as lymphoid organs and blood [27–29]. PMCA offers the possibility of amplifying the amount of PrP^{Sc} in a sample, making its detection by existing methods easier. Thus, a strategy that combines prion amplification *in vitro* with highly sensitive detection methods should allow early diagnosis of TSE. The aim would be not only to detect prions in the brain in early pre-symptomatic cases, but also to generate a test to diagnose living animals and people.



A crucial issue in TSE is the unprecedented nature of the infectious agent which, according to the prion hypothesis, is composed of a single protein that propagates in the absence of nucleic acid [11]. Although, strong evidence in favor of this hypothesis has been reported in the last few years, it is still a matter of controversy among many scientists [10,30]. Perhaps the most important evidence for the protein-only hypothesis that has not yet been obtained is the generation of infectivity in the test tube [10]. PMCA provides a unique opportunity to evaluate the infectious properties of PrP^{Sc} generated *in vitro* because, after amplification under optimal conditions, >99% of protease-resistant protein is composed of newly produced PrP^{Sc}. The high yield following conversion is essential to distinguish newly generated infectivity from that used to initiate the reaction.

Based on data from transgenic animals, it has been proposed that additional brain factors present in the host are essential for prion propagation [31]. We demonstrated previously that prion conversion does not occur under our experimental conditions when purified PrP^C and PrP^{Sc} are mixed and incubated [32]. The conversion activity was recovered when the bulk of cellular proteins were added back to the sample [32]. This finding provides direct evidence that other factors present in the brain are essential to catalyze prion

propagation. PMCA could be useful as a biochemical assay to identify these conversion factors.

Beyond prion disease

Perhaps the most exciting research for coming years will be to understand whether the prion phenomenon of propagation of biological information is exclusively associated with a limited group of proteins (e.g. PrP) or is a more general process in biology. The findings of proteins with a prion-like behavior in yeast and other fungi have provided a step forward in this direction [33,34]. It seems possible that transmission of protein conformation from one molecule to another might represent a natural process to modify, within one generation, the structure and activity of proteins to adapt to new conditions. The possibility that many proteins adopt multiple conformations to exert different functions, and that this biological information can be propagated between different individuals, might revolutionize our understanding of biology. We cannot predict precisely how common the prion phenomenon is in nature, but being able to reproduce it *in vitro* with high efficiency could give us a powerful tool to explore the amplification of protein (mis)folding using proteins not yet known to have prion behavior.

At present, it has not been shown that any of the other PCD have an infectious origin, or that their misfolded protein is

able to infect an individual and induce conversion of the natively folded protein. However, in a similar way to PrP^{Sc} in TSE, the protein conformational changes associated with the pathogenesis of these diseases result in formation of abnormal proteins rich in β -sheet structure, partially resistant to proteolysis and with a high tendency to aggregate [3,7,8]. Indeed, a common feature of several PCD (including TSE) is the aggregation and deposition of the misfolded protein in different organs in the form of amyloid plaques. The available data indicate that amyloid deposition follows a seeding-nucleation mechanism [21] (Fig. 3). Analogous to a crystallization process, amyloid formation depends on the slow interaction between misfolded protein monomers to form oligomeric nuclei, around which a faster phase of elongation takes place (Fig. 3). The limiting step in this process is nucleus formation, and the extent of amyloidosis depends on the number of seeds produced [21,35].

The conditions used to produce amyloid fibrils *in vitro* have been optimized for most of the proteins implicated in the formation of amyloid plaques [8,36]. However, the experimental procedures usually involve highly concentrated and non-physiological protein solutions to speed up the nucleus formation and induce aggregation in a short time. It is likely that a small amount of misfolded oligomer is present in tissues and biological fluids of individuals much before the appearance of clinical symptoms. These endogenous oligomeric nuclei could be used to induce the aggregation of seed-free, low concentrations of the amyloid protein. Combining phases of incubation (to allow protein-protein interaction resulting in the elongation of seeds) with phases of sonication (to multiply the number of nuclei) should produce a cyclic amplification of amyloid formation. Thus, the principles of PMCA could be used to detect low concentrations of early intermediates of amyloid formation in tissues or biological fluids of people incubating some amyloid-related disorders. Therefore, PMCA could have broader applications for research and diagnosis of diseases in which protein misfolding and aggregation is implicated.

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Claudio Soto*

15 Chemin des Mines, 1211 Geneva, Switzerland.

*e-mail: claudio.soto@serono.com

Gabriela P. Saborio

Laurence Anderes

Serono International SA, Geneva, Switzerland.